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[COMMUNICATION]

Involvement of a Serine Protease in the Activation of Prophenoloxidase in *Drosophila melanogaster*

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ABSTRACT—Prophenoloxidase (ProPO) in the crude preparation was activated by the fraction containing activating components (designated as AMM-1) prepared from mature third instar larvae. The activation could effectively be inhibited by *p*-nitrophenylguanidinobenzoate (*p*-NPGB) and *p*-amidinophenylmethanesulfonate (*p*-APMSF) at low concentrations. In AMM-1, tryptic activity was demonstrated and this activity could be suppressed by above inhibitors at the concentrations comparable to those inhibiting ProPO activation. These facts strongly suggest that a serine protease of trypsin type is involved in the activation prosess of ProPO in *Drosophila melanogaster*.

INTRODUCTION

In insect hemolymph, phenoloxidase (PO) occurs as an inert zymogen (prophenoloxidase, ProPO) and it can be activated upon bleeding [1]. A number of studies have been reported on the mechanism of the activation. However, no unified principle as to the activating mechanism has yet been obtained.

In *Drosophila*, activation of ProPO was first reported by Ohnishi [2]. Since then, many authors conducted experiments on various aspects of this phenomenon. Among them, Mitchell's group conducted most extensive biochemical studies on the activation and they proposed a cascade mechanism involving at least 6 components [3]. However, the nature of the reactions of these components remained unsolved.

In *Bombyx mori*, Dohke reported with his purified ProPO-activating enzyme from larval cuticles that the enzyme exhibited proteolytic activity and could be classified as a serine protease of trypsin type [4]. Further studies on this material by Ashida's group have revealed that a limited proteolysis of ProPO is involved in the activation by this enzyme, resulting in a release of a peptide [5, 6].

In the present communication, we have investigated the possibility of the participation of protease in the activation process of ProPO in *Drosophila melanogaster*.

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MATERIALS AND METHODS

Insects

Oregon-R strain was reared on standard cornmeal-yeast medium of Bowling Green formula at 25°C±0.5. Mature third instar larvae were collected, frozen with liquid nitrogen and stored at −80°C until use.

Reagents

Benzoyl-arginine ethylester (BAEE) and benzoyl-*p*-nitroanilide (BANA) were purchased from Peptide Institute Inc. *L*-Dopa and *p*-NPGB were from Sigma. *p*-APMSF was from Wako Pure

Chem. Industries Ltd.

Preparation of activating components (AMM-1)

Preparation of the activating components, designated as AMM-1 by Seybold and his collaborators, was made according to their description [3]. Essentially, it consisted of the fraction precipitable with ammonium sulfate at 35% saturation. Supernatant of the 35% saturated ammonium sulfate was saved for the preparation of crude ProPO as described below.

Preparation of crude ProPO

Crude ProPO was prepared by the same method as that of "crude A_1" of Seybold *et al.* [3], except that the starting material was mature third instar larvae. Essentially, the preparation consisted of the fraction precipitable with ammonium sulfate from 44% to 56% saturation.

Assay of PO

PO activity was assayed by the method of Horowitz and Shen [7], with the following modifications. *L*-Dopa was used instead of *DL*-dopa and microtubes with microtube adaptor were employed for Klett-Summerson photometer.

Assay of tryptic activity

Tryptic activity was assayed with BAEE and BANA. Increase in absorbancy at 254 nm (BAEE) or 405 nm (BANA) was followed at intervals after incubation at 25°C of the enzyme preparation with the substrate in 40 mM Tris-HCl buffer, pH 7.8 [8].

RESULTS AND DISCUSSION

Crude ProPO fraction could be obtained from extracts by precipitation with ammonium sulfate between 44 and 56% saturation. The fraction containing the activating components (AMM-1) could be obtained by precipitating the proteins with ammonium sulfate at 35% saturation. Upon incubation of a mixture of both fractions at 0°C, PO activity appeared immediately and increased rapidly (Fig. 1). The activating activity of AMM-1 could be suppressed at low concentration of *p*-NPGB, which has been known to be a titrant of

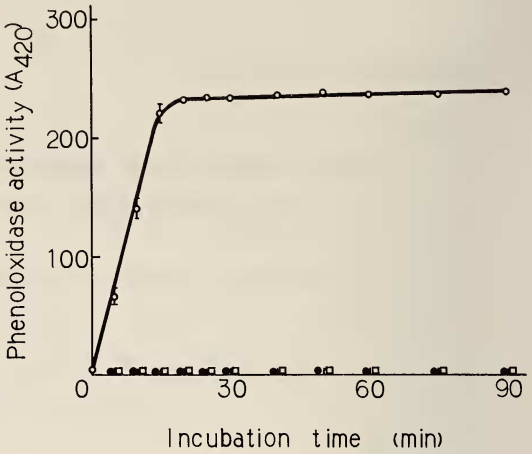

FIG. 1. Effect of *p*-NPGB on the activation of prophenoloxidase.
One part of crude ProPO preparation (protein 8.94 mg/ml) was mixed with one part of AMM-1 (protein 2.33 mg/ml) and the mixture was incubated at 0°C. At intervals, aliquot was sampled for PO assay. ProPO was incubated without AMM-1 (●—●), with AMM-1 (○—○), with AMM-1 preincubated with 100 μM *p*-NPGB at 0°C for 1 hr (□—□). The vertical bars represent the standard error of the mean, respectively. PO activity is expressed arbitrarily by Klett units.

trypsin and was reported to inhibit ProPO-activating enzyme from *Bombyx mori* cuticles [4]. The activating activity was also inhibited by *p*-APMSF, which is a potent inhibitor of serine protease [9].

If some component(s) of the cascade critical to the activation is serine protease of trypsin type, the tryptic activity could be demonstrated in the AMM-1 fraction. This prediction was verified by the experiments with synthetic substrates. When AMM-1 was incubated at 25°C with BAEE or BANA, esterase as well as amidase activity could be demonstrated (Table 1). Furthermore, this activity could be suppressed by *p*-NPGB and *p*-APMSF at the concentrations comparable to those inhibiting the ProPO activation. If the protease of trypsin type participates the activation reaction, it is also predicted that peptidic substrates such as BAEE or BANA could inhibit the activation competitively. The result of the experiment in which BAEE was included at the concentration of 10^{-3} M revealed 41.7% inhibition, supporting above prediction. These facts indicate that a serine

TABLE 1. Effect of proteinase inhibitors on tryptic and activating activity of AMM-1

Substrate	Inhibitor (μM)		Activity (%)
BANA	*p*-NPGB	0.1	48.0
		10	22.3
	p-APMSF	1.0	61.1
		100	23.6
BAEE	*p*-NPGB	0.1	91.2
		10	11.0
	p-APMSF	1.0	69.0
		100	17.1
ProPO	*p*-NPGB	0.1	63.3
		10	9.8
	p-APMSF	1.0	63.4
		100	30.9

For the tryptic activity, AMM-1 (protein 3.75 mg/ml) was preincubated with the inhibitor at 0°C for 1 hour and then the activity was estimated. For the activating activity, AMM-1 was mixed with ProPO (protein 12.35 mg/ml) at 0°C for the activation and PO activity was assayed 5 minutes after the addition of AMM-1.

protease of trypsin type is involved in the activating process of ProPO in *Drosophila melanogaster*.

According to Seybold and his collaborators, the cascade reaction responsible for the activation of ProPO in *Drosophila* is as follows:

$$\text{PreS} \xrightarrow{\text{S-activator}} \text{S}$$

$$\text{P} \xrightarrow{\text{S}} \text{P}'$$

$$\text{P}' + \text{A}_1, \text{A}_2, \text{A}_3 \longrightarrow \text{active PO}$$

They did not comment on the nature of the reactions in the cascade.

Consistent with our finding is the report by Ashida and his group on the prophenoloxidase activation in the mosquito, *Aedes aegypti* L. that the activation in the crude preparation from whole larvae could effectively be suppressed by *p*-NPGB and *p*-APMSF [10].

In the housefly, *Musca vicina*, we showed that the activation of prophenoloxidase (protyrosinase) was catalyzed by the activator of protein nature and that the concentration of the activator increased during the course of activation [11]. Using the same materials, Funatsu and his collaborators made extensive studies on the phenoloxidase system. They have shown that the activation involves association-dissociation of subunits [12]. However, the role of the natural activator has remained unsolved. They have previously demonstrated by their kinetic studies that the natural activator behaved as an enzyme acting on the prophenoloxidase as the substrate [13].

Thus even in dipterans, we have no unified theory on the mechanism of the activation. We assume that most, if not all, of the components of the activation cascade are serine proteases, the last one in the cascade being trypsin type. Experiments to prove this hypothesis are now in progress.

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